

Nicotine inhibits the metabolic activation of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in rats*

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The effect of nicotine on the metabolism of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) was studied in rats. [1-¹⁴C]NNK was s.c. injected at a dose of 0.08 μ mol/kg. Co-administration of a 500-fold higher dose of nicotine (40 μ mol/kg) did not reduce the overall urinary excretion of radioactivity. However, the metabolic pattern in 24 h urine was significantly changed. Metabolites resulting from NNK activation by α -hydroxylation were significantly ($P < 0.001$) reduced to 72% of the control. Detoxification to *N*-oxides and the glucuronide of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol increased to 155% ($P < 0.01$) and 188% ($P < 0.01$) of the control respectively. These results suggest that nicotine, which occurs in concentrations up to 30 000-fold higher than NNK in mainstream smoke of cigarettes may have a protective effect against metabolic activation of NNK.

Introduction

The tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK*) has been suggested to be involved in the induction of lung cancer in smokers (1,2). NNK is a potent pulmonary carcinogen and also induces tumors of the nasal mucosa, exocrine pancreas and liver in rats (1-3). Together with *N*-nitrosomonicotine (NNN), it may be involved in the etiology of oral cancer in users of smokeless tobacco products (2).

NNK requires metabolic activation for expression of its carcinogenicity. Studies in rodents conclusively demonstrate that hydroxylation of the methylene and methyl carbons adjacent to the *N*-nitroso group (so-called α -hydroxylation) are the key metabolic processes leading to the initiation of carcinogenesis (1,4-9). As shown in Figure 1 the common urinary metabolite from these two reactions is 4-oxo-(3-pyridyl)butyric acid (keto acid). The product of NNK carbonyl reduction, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) is also a rodent carcinogen (4,10). NNAL undergoes α -hydroxylation in a similar way to NNK resulting in the formation of the common urinary metabolite, 4-hydroxy-(3-pyridyl)butyric acid (hydroxy acid). Detoxification pathways include glucuronidation of NNAL and pyridine-*N*-oxidation of both NNK and NNAL. All these

*Abbreviations: NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*-nitrosomonicotine; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; hydroxy acid, 4-hydroxy-(3-pyridyl)butyric acid; keto acid, 4-oxo-(3-pyridyl)butyric acid; NNAL-Gluc, [4-(methylnitrosamino)-1-(3-pyridyl)-but-1-yl]- β -D-glucopyranosiduronic acid; NNAL-*N*-oxide, 4-(methylnitrosamino)-1-(3-pyridyl)-*N*-oxide-1-butanone; NNK-*N*-oxide, 4-(methylnitrosamino)-1-(3-pyridyl)-*N*-oxide-1-butanone; HPB, 4-hydroxy-(3-pyridyl)-1-butanone.

*Dedicated to Professor Dr R. Preussmann on the occasion of his 65th birthday.

metabolic pathways have been demonstrated not only in rodent species (1,4,10-13), but also in primates (14-16) and in human tissues (17-19). In humans the presence of hemoglobin and DNA adducts formed from NNK and/or NNN has been reported (20, 21). Recently, the NNK metabolites NNAL and its glucuronide have been detected in 24 h urine of smokers (22).

The tobacco alkaloid nicotine, quantitatively the main component of cigarette smoke, is present at 3000- to 30 000-fold higher concentrations than NNK in the mainstream and sidestream smoke (23,24). In rat oral tissue, nicotine inhibits the metabolic activation of NNK when present at 100-fold higher concentrations than NNK (25). For the rabbit olfactory-specific cytochrome P450 isozyme NMa, nicotine is a competitive inhibitor of NNK α -hydroxylation (26). The only *in vivo* study using a NNK dose far in excess of that of nicotine did not show any effect of nicotine on NNK activation (27). Therefore, we investigated the metabolism of NNK in the rat when given together with a 500-fold higher dose of nicotine.

Materials and methods

Chemicals

[1-¹⁴C]NNK with a sp. act. of 29 mCi/mmol was obtained from Chemsyn Science Laboratories (Lenexa, KS). NNK metabolite standards were a gift from Dr D. Hoffmann (American Health Foundation, Valhalla, NY). (-)-Nicotine, β -glucuronidase (type IX) and thimerosal (sodium ethylmercurithiosalicylate) were obtained from Sigma Chemie GmbH (Taufkirchen, Germany). All other chemicals, which were of either HPLC or analytical grade, were purchased from Merck (Darmstadt, Germany).

Animals

Male Wistar rats (85-110 g) from the breeding colony of the University of Göttingen were housed in stainless steel metabolism cages in a fully air-conditioned room (18 \pm 1°C; 60 \pm 5% humidity). The day-night cycle was 12 h (light from 7 a.m.). The animals had unrestricted access to Alma H 1003 Laboratory chow (F. Botzenhardt KG, Kempten, Germany) and drinking water. The animal experiments were officially approved by the Government of Upper Bavaria (AZ 211-2531-53/92).

Collection of rat urine

Groups of eight rats were administered 0.4 ml of saline with either [1-¹⁴C]NNK (8 nmol = 1.66 μ g) alone or [1-¹⁴C]NNK plus nicotine (4 μ mol = 650 μ g) by s.c. injection. Urine was collected over 24 h time intervals for 2 days in polyethylene vials containing a few grains of thimerosal. Aliquots of 100 and 500 μ l from the first and second day of the experiment respectively were used for total ¹⁴C determination. The remaining urine was centrifuged and the supernatant stored at -20°C until analysis by HPLC.

HPLC analysis

Urine samples from the first 24 h of the experiment were chromatographed on a 4.6 \times 250 mm LiChrosorb® RP18 SelectB column (Merck) by elution with a gradient of 100% A for 0.5 min, linear to 80% A/20% B in 20 min and linear to 20% A/80% B in 2 min (A: 20 mM Tris buffer, pH 7.2; B: acetonitrile) at a flow rate of 0.7 ml/min. Detection of ¹⁴C was performed by solid-phase radioactivity monitoring (Ramona, Raytest, Straubenhardt, Germany). Radioactive metabolites were identified by co-chromatography with unlabeled reference compounds detected by UV at 234 and 254 nm (UVD 160, Gynkoteck, Germering Germany). The *O*-glucuronide of NNAL was further characterized by co-injecting the purified radioactive compound obtained in a previous study (12) and by treatment of urine with β -glucuronidase (13).

Statistical analysis

Reported values represent means \pm standard error. Statistical analysis was performed by the two-sided *t*-test for independent samples.

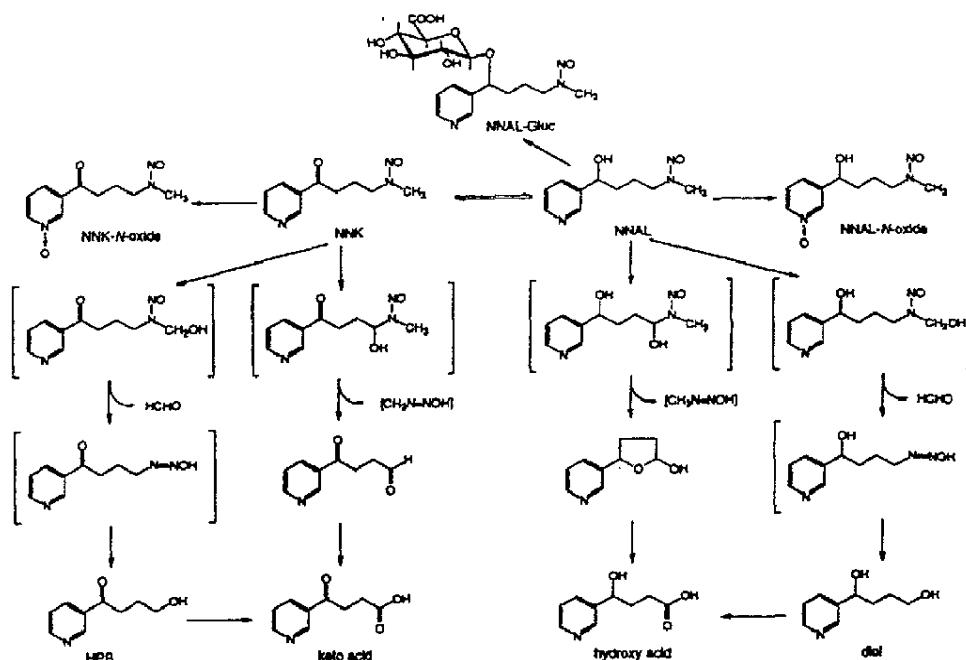


Fig. 1. Metabolic scheme of NNK. Structures in brackets are hypothetical intermediates (13).

Results

The urinary excretion of NNK was studied in rats s.c. injected with ~80 nmol/kg NNK either alone or in combination with ~40 µmol/kg nicotine. This represents a 500-fold higher dose of nicotine than NNK. In excess of 98% of the total urinary excretion occurred within the first 24 h with no difference between NNK only ($72.4 \pm 2.9\%$) and NNK plus nicotine ($74.8 \pm 2.1\%$) treated rats.

Figure 2 shows typical HPLC profiles of the urinary metabolites of [1-¹⁴C]NNK obtained from rats treated with or without a 500-fold excess of nicotine. Peaks I and II co-eluted with hydroxy acid and 4-oxo-4-(3-pyridyl)butyric acid (keto acid) resulting from α -hydroxylation of NNK and NNAL respectively (Figure 1). Peak III corresponds to [4-(methylnitrosamino)-1-(3-pyridyl)-but-1-yl]- β -D-glucopyranosiduronic acid (NNAL-Gluc). Peaks IV and V co-eluted with 4-(methylnitrosamino)-1-(3-pyridyl)-N-oxide-1-butanol (NNAL-N-oxide) and 4-(methylnitrosamino)-1-(3-pyridyl)-N-oxide-1-butanone (NNK-N-oxide). 4-Hydroxy-1-(3-pyridyl)-butanol (diol), 4-oxo-1-(3-pyridyl)-1-butanone (HPB) and NNAL were occasionally detected. Of these three metabolites, only NNAL exceeded 2% of the radioactivity in five and two of eight samples of 24 h urine of the NNK only and NNK plus nicotine-treated rats respectively.

Figure 3 shows the pattern of the five major NNK metabolites in 24 h urine. Nicotine treatment significantly reduced the formation of hydroxy acid and keto acid to 75% ($P < 0.001$) and 69% ($P < 0.001$) of the control respectively. The detoxification products NNAL-Gluc and NNAL-*N*-oxide were significantly increased by 188% ($P < 0.01$) and 163% ($P < 0.001$) of the control respectively by co-administration of nicotine. The formation of NNK-*N*-oxide was also increased 124% but the difference did not reach statistical significance. In Table I the results are presented as total amounts excreted in 24 h urine.

Discussion

Cigarette smoke contains several thousand different components, of which nicotine is quantitatively the most abundant. Nicotine occurs in concentrations 3000- to 30 000-fold higher than NNK in mainstream cigarette smoke (23,24). As such, nicotine was chosen as the first candidate with which to study the *in vivo* inhibition of NNK metabolism. Previous *in vitro* studies have shown that nicotine is a potent competitive inhibitor of NNK α -hydroxylation in hamster lung (28), rat oral tissue (25) and in rabbit nasal olfactory and respiratory microsomes (26). The effect of multiple-dose exposure to nicotine on the *in vivo* metabolism of NNK in rats (27) and *in vitro* pulmonary metabolism of NNK in hamsters (29) has also been studied. In both studies, 0.002% nicotine was administered in drinking water for 14 days. After nicotine pretreatment, no inhibition of *in vivo* metabolic activation of NNK was observed in rats given a single i.v. dose of 0.4 mmol NNK/kg body wt (27). Contrary to this finding, nicotine pretreatment of hamsters induced pulmonary α -hydroxylation in lung explants (29). In both studies, the nicotine dose was 2000- to 20 000-fold lower than that of NNK.

In the reported study, the nicotine dose was 500-fold higher than that of NNK. Subcutaneous injection of both nicotine and NNK was chosen as the route of administration. Following s.c. injection, NNK is first transported to the lung, which is considered to be the primary site of NNK metabolism. Previous studies using *N*-nitrosodibutylamine have shown a high first-pass metabolism in the lung following either s.c. or i.v. administration (30). Numerous studies with isolated perfused rat lung (31) as well as different *in vitro* preparations of rat lung (5,9,11,32–36) have demonstrated extensive metabolism of NNK by α -hydroxylation, *N*-oxidation and reduction to NNAL in this organ. Quantitative comparison of urinary NNK metabolites in Wistar rats clearly shows nicotine inhibition of the metabolic activation of this

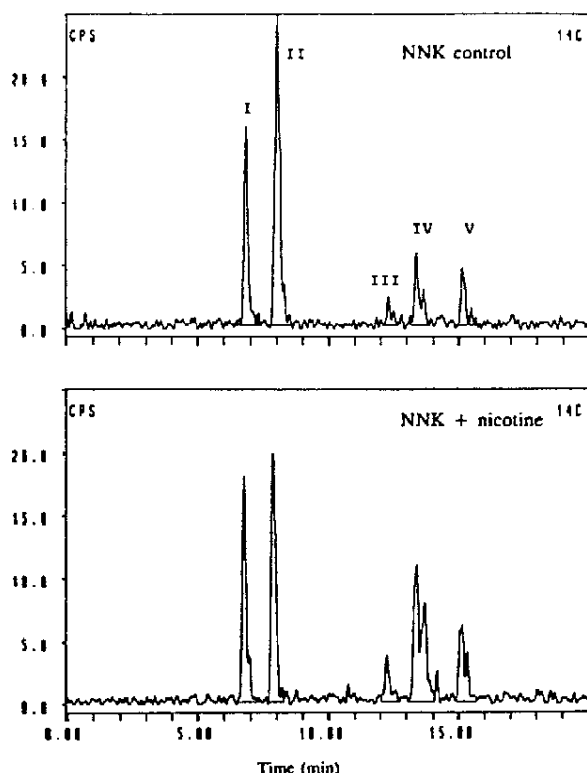


Fig. 2. HPLC analysis of metabolites in the urine of rats 24 h following s.c. administration of [1-¹⁴C]NNK at 80 nmol/kg with or without nicotine at 40 μ mol/kg. Labeling of radioactive peaks—hydroxy acid (I), keto acid (II), NNAL-Gluc (III), NNAL-*N*-oxide (IV) and NNK-*N*-oxide (V)—refers to peaks co-eluting with authentic standards added as UV markers.

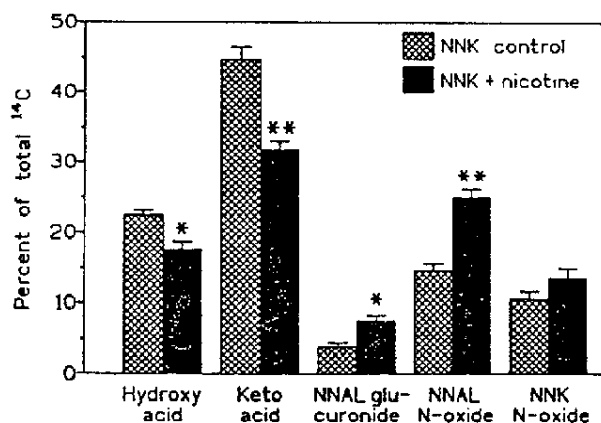


Fig. 3. Effect of nicotine on the metabolite pattern in 24 h urine following s.c. administration of [1-¹⁴C]NNK at 80 nmol/kg with or without nicotine at 40 μ mol/kg. Values are the mean \pm SE of eight samples. Asterisks indicate a statistically significant difference to the group given only NNK at * P < 0.01 and ** P < 0.001.

tobacco-specific nitrosamine (Table I). The largest suppression was observed for the keto acid, which results from α -hydroxylation of NNK (Figure 1). This decrease was not compensated by

Table I. Effect of nicotine on excretion of NNK metabolites in 24 h urine^a

Metabolite	pmol detected in urine (% of urinary metabolites)			
	NNK		NNK + nicotine	
Hydroxy acid	668 \pm 17	(22.4) ^d	502 \pm 24	(17.6)***
Keto acid	1332 \pm 50	(44.7)	919 \pm 35	(31.9)***
NNAL-Gluc	118 \pm 20	(3.9)	222 \pm 26	(7.6)**
NNAL- <i>N</i> -oxide	441 \pm 38	(13.3)	719 \pm 27	(25.0)***
NNK- <i>N</i> -oxide	320 \pm 37	(10.6)	397 \pm 54	(13.5)
Σ of alpha ^b	2021 \pm 57	(67.8)	1462 \pm 41	(50.8)***
Σ of <i>N</i> -oxides	720 \pm 79	(23.8)	1116 \pm 68	(38.5)**
NNAL-Gluc + NNAL ^c	161 \pm 21	(6.2)	296 \pm 41	(10.1)*
Total	2902 \pm 104	(96.9)	2874 \pm 105	(99.3)

^aGroups of eight male Wistar rats were administered [1-¹⁴C]NNK at a dose of 80 nmol/kg with or without nicotine at a dose of 40 μ mol/kg. Urine was collected for 24 h and analyzed for urinary metabolites of NNK as indicated in the text.

^bIncluding hydroxy acid, keto acid, diol (detected in one sample from each experiment) and HPB (detected in four and five samples from experiments with NNK and NNK + nicotine respectively).

^cNNAL was detected in five and six samples from experiments with NNK and NNK + nicotine respectively.

^dMean \pm SE of eight samples. Values labeled are statistically significantly different from the NNK group at * P < 0.05, ** P < 0.01 and *** P < 0.001.

a comparable increase in NNK-*N*-oxide formation. Therefore, the *in vivo* equilibrium existing between NNK and NNAL as described by Adams *et al.* (15) is shifted in favor of NNAL. The urinary excretion of hydroxy acid resulting from α -hydroxylation of NNAL was also reduced. The excretion of NNAL and its detoxification products, NNAL-Gluc and NNAL-*N*-oxide, expressed as total urinary excretion of ¹⁴C nearly doubled from 19.9 \pm 1.5% in control rats to 35.1 \pm 1.0% in rats treated with nicotine.

Whether nicotine specifically inhibits metabolic activation of NNK by α -hydroxylation at either the methylene or methyl carbon atom adjacent to the *N*-nitroso group or both sites simultaneously, cannot be determined from the present results. Methylene hydroxylation produces methanediazohydroxide, which can methylate DNA bases *in vivo*. Methyl hydroxylation yields 4-(3-pyridyl)-4-oxobutanediazohydroxide, which pyridyl-oxobutylates DNA. Which of these two pathways plays the predominant role in the proposed carcinogenic effect of NNK in humans is unknown (37). The extent of pyridyloxobutylation of DNA by NNK can be estimated by measuring HPB released by alkaline hydrolysis of hemoglobin (38). This HPB-releasing adduct is also formed by NNN (39) and has been quantified in tobacco users, since it is considered to be a surrogate marker for the uptake and activation of both NNN and NNK (20). Such studies show a <3-fold difference in HPB-releasing hemoglobin adduct levels in smokers and nonsmokers (20,40,41). The results of the present study clearly show that nicotine inhibits NNK α -hydroxylation, the required metabolic step in the formation of HPB-releasing adducts.

Additional comparative studies need to be performed with NNN, both NNN and NNK in combination and with a higher nicotine dose relative to both nitrosamines to determine the full extent of nicotine inhibition of tobacco-specific nitrosamine metabolism. Furthermore, the mechanisms of nicotine inhibition of NNK metabolism and both hemoglobin and tissue adduct formation need to be studied.

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